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Comprehensive analyses reveal molecular and clinical characteristics of RNA modification writers across 32 cancer types

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ABSTRACT

Adenosine alterations to RNA, which are largely determined by RNA modification writers (RMWs), are critical for cancer growth and progression. These RMWs can catalyze different types of adenosine modifications, such as N6-methyladenosine (m6A), N1-methyladenosine (m1A), alternative polyadenylation (APA), and adenosine-to-inosine (A-to-I) RNA editing. These modifications have profound effects on gene expression and function, such as immune response, cell development. Despite this, the clinical effects of RMW interactive genes on these cancers remain largely unclear. A comprehensive analysis of the clinical impact of these epigenetic regulators in pan-cancer requires further comprehensive exploration. Here, we systematically profiled the molecular and clinical characteristics of 26 RMWs across 33 cancer types using multi-omics datasets and validated the expression level of some RMWs in various cancer lines. Our findings indicated that a majority of RMWs exhibited high expression in diverse cancer types, and this expression was found to be significantly associated with poor patient outcomes. In the genetic alterations, the amplification and mutation of RMWs were the dominant alteration events. Consequently, the RNA Modification Writer Score (RMW score) was established as a means to assess the risk of RMWs in pan-cancer. We found that 27 of 33 cancers had significantly higher scores compared with normal tissues, and it was significantly correlated with prognosis. We also evaluated their impact on the tumor microenvironment and the response to immunotherapy and targeted therapy. These findings verified the important role of RMWs in different aspects of cancer biology, and provided biomarkers and personalized therapeutic targets for cancer.

KEYWORDS

RNA modification writer; pan-cancer; genomics; prognosis; tumor immune microenvironment

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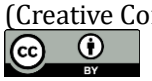

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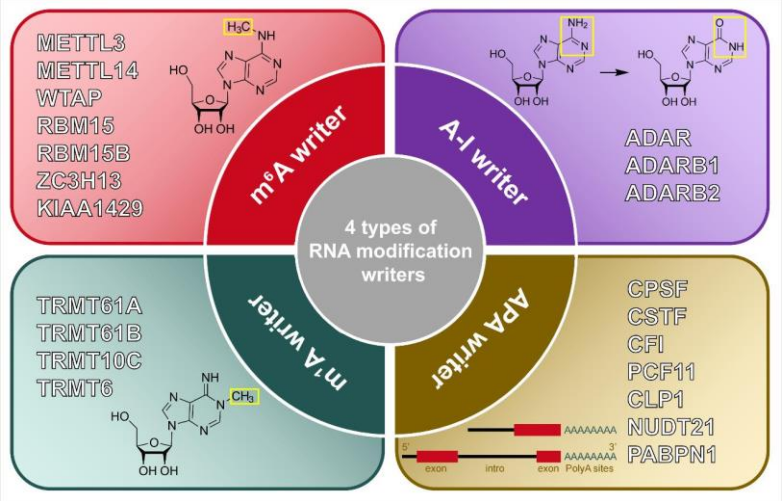
Graphical abstract



Comprehensive analysis of molecular and clinical characteristics of RMW sin pan-cancer.

1. Introduction

RNA modification is an epigenetic alteration that exerts influence over various facets of the RNA metabolism, such as splicing[1], translation[2], stability[3], and localization[4], which enhance the diversity of RNA molecules, thereby broadening functional capacities. It has been found that 140 kinds of RNA modifications are present across all fields of life, which are essential for gene regulation. Among the different types of RNA modifications, adenosine alterations are the most abundant and diverse and are mediated by a group of enzymes known as RNA modification writers (RMWs)[5]. These RMWs can catalyze different types of adenosine modifications, such as N6-methyladenosine (m6A)[6], N1-methyladenosine (m1A)[7], alternative polyadenylation (APA)[8] and adenosine-to-inosine (A-to-I) RNA editing[9]. These modifications can have profound effects on gene expression and function, such as immune response[10], cell differentiation[11], development[12], and stress response[13]. Much literature has reported the relationship between these modifications and various diseases, especially cancer, several cancer types have been linked to m6A methylation modifications, including breast cancer, liver cancer, gastric cancer, and lung cancer[13-15].

The function of m6A methylation writers is to add methyl to adenosine N6 position in mRNA, thus becoming a dynamic and abundant RNA modification [15]. The core members of m6A writers are METTL3, METTL14, WTAP and KIAA1429 [16], which cooperate to recognize and methylate specific RNA targets. Other accessory proteins, such as RBM15/15B and ZC3H13, can modulate the activity or specificity of the core complex[17]. m6A methylation writers played important roles in diverse functions, such as embryonic development[18], neurogenesis[19], immune response[20], cell differentiation[21], drug resistance and tumorigenesis[22]. For example, METTL3 suppresses the growth and metastasis of hepatocellular carcinoma by inhibiting SOCS expression[23]. METTL14 activated MYC in acute myeloid leukemia to promote tumor[24].

m1A methylation writers create an abundant and evolutionarily conserved RNA modification[25]. The known m1A methylation writers are TRMT61A, TRMT61B, TRMT10C and TRMT6[26], which belong to the TRM family of tRNA methyltransferase. m1A methylation writers regulate the stability, structure and function, by affecting their interactions with ribosomes[7], translation factors[27] and RNA-binding proteins[28]. The role of m1A methylation writers was studied less than m6A methylation writers in cancers, but some evidence suggests that they may have tumor-promoting[29] or tumor-inhibiting effects[30] depending on the cancer type.

APA writers catalyze the alternative cleavage and polyadenylation (APA) of transcripts, which produce diverse untranslated regions (UTRs) and poly A tails[31]. The known APA writers are CPSF, CSTF, CFI, PCF11, CLP1, NUDT21 and PABPN, which belong to the cleavage and polyadenylation specificity factor complex[32]. APA writers regulate the stability[33], translation[34], and localization[35] of mRNA transcripts, by affecting their interactions with microRNAs[36], RNA-binding proteins[37] and RNA decay[38] factors. According to previous reports, APA writers regulated in many biological pathways[35,39-41]. For example, CSTF regulated the expression of TP53 by producing a longer 3' UTR that enhances its stability[42].

A-I RNA editing writers -- ADAR, ADARB1, and ADARB2, which belong to the adenosine deaminase acting on RNA (ADAR) family of enzymes[43], deaminate adenosine to inosine[44]. A-to-I RNA editing writers regulate the splicing[45], structure[46], and function[47] of RNA molecules, by changing their base-pairing properties and interactions with RNA-binding proteins. A-I RNA editing writers can change the function by editing the bases in the coding[48] or non-coding[49] regions of oncogenes or tumor suppressors. For example, ADAR1 edited the coding region of AZIN1, resulting in a function acquired mutation that promotes the growth and migration of hepatocellular carcinoma cells[50], however, lncRNA LINC00624 promoted tumor progression and drug resistance by stabilizing ADAR1[50].

In summary, RMWs play critical roles in cancer[51,52]and response to therapy[53]. Therefore, it is important to understand the expression, mutation, regulation, and function of RMWs across different cancer types and their implications for cancer prognosis and therapy. However, the molecular and clinical characteristics of RMWs across different cancer types are still largely unexplored. In this study, we systematically profiled the expression and mutation of 26 RMWs across 32 cancer types using multi-omics data. We investigated the associations between RMWs and tumor molecular subtypes, survival outcomes, immune infiltration, drug sensitivity, and therapeutic efficacy. We also constructed the RMW score to evaluate the risk of different subtypes of tumors, the impact of RMWs on tumor microenvironment and the response to immunotherapy and targeted therapy. Our study revealed the molecular and clinical characteristics of RMWs in pan-cancer, and provided potential biomarkers and therapeutic targets for personalized cancer treatment.

2. Methods and materials

2.1. Differential expression of RMW genes

RNA -Seq data of pan-cancer were obtained from GTEX and TCGA datasets and downloaded from UCSC Xena website (https://xena.ucsc.edu/). Differential gene expression analysis was performed with R package limma. P-values and logFC of the RMW genes were extracted for heatmap display with R package ggplot2.

2.2. Survival analysis of RMW genes

Gene expression of pan-cancer for the GTEx and TCGA datasets, assaying via RNA-seq, was downloaded from the UCSC Xena Portal. Differential gene expression analysis was performed with R package limma. The p-values and logFC of the RMW genes were extracted for heatmap display with R package ggplot2.

2.3. Construction of risky score

The number of genes with significance of Cox analysis in pan-cancer was recorded. If the p value was < 0.05, hazard ratio (HR) >1, the gene was taken as a risk factor and the score was added one point; Conversely, if the p value was < 0.05, HR <1, the gene is awarded for the protection factor and the score was reduced one point. In summary, the final score was used as a risky score. The correlation between RMWs in pan-cancer was shown by heatmap.

2.4. Validation and mutation of RMW genes

Validation of RMWs in pan-cancer was analyzed by the online website cBioPortal (https://www.cbioportal.org/), including mutations, fusions, amplifications, deletions as well as multiplex variations.

2.5. Gene copy number variation analysis of RMW

Data on CNV percentage in each cancer, a correlation between CNV with gene mRNA expression, and the profile of heterozygous CNV and homozygous CNV were from the online website GSCA database (https://guolab.wchscu.cn/GSCA/#/).

2.6. Methylation analysis of RMW

Correlation between methylation and expression and methylation difference between tumor and adjust normal samples were analyzed by online website GSCA database.

2.7. Gene regulatory and protein interaction network construction

Transcription factors and miRNA upstream of the RMWs gene were analyzed using the Regnetwork (https://regnetworkweb.org/) and the protein interaction network was analyzed using the STRING (https://cn.string-db.org/). Networks were visualized using the Cytoscape software.

2.8. Construction of RMW score

The RMW score was analyzed by ssGSEA of the R package GSVA. Boxplots of the increase in RMW score of 33 tumors from left to right and differences between tumors and paired normal tissues were plotted by R package ggplot2.

2.9. Survival analysis by RMW score in pan-cancer

Four types of survival analysis models were analyzed by univariate Cox regression in pan-cancer with R package survminer and survival, then, plots were plotted with R package forestplot.

2.10. The GSVA analysis of the RMW score

Correlation between the RMW score and pathways was calculated in each tumor separately and heatmap was plotted with R package pheatmap. The gene sets of GSVA analysis were the latest MsigDB database hallmark gene sets.

2.11. Tumor microenvironment analysis of RMW score

Stromalcore, ImmuneScore, ESTIMATEScore, and TumorPurity were calculated by R package ESTIMATE and plotted by ggplot2 according to previous research methods[54].

2.12. Immune cell infiltration analysis of RMW score

Immune-infiltration data came from the ImmuCellAI database (https://guolab.wchscu.cn/ImmuCellAI/#!/) and TIMER2 database (http://timer.comp-genomics.org/timer/), a heatmap was plotted by R package ggplot2.

2.13. Analysis of immune related genes correlation in pan-cancer

Correlation between immunoactivated genes, MHC genes, chemotactic factor genes, chemokine receptor genes, and RMW score was shown as a heatmap plotted by R package ggplot2.

2.14. Prognosis analysis of patients with immunotherapy and targeted therapy

The prognostic information of patients after different treatments comes from the previous literature, GSE135222[55] and GSE176307[56]. Survival analysis was performed using the R package survival, plotted using the R package ggplot2.

2.15. Drug sensitivity analysis of RMW in pan-cancer

The IC_{50} data of drug sensitivity of RMWs were from t the Gene Set Cancer Analysis database (GSCA, http://bioinfo.life.hust.edu.cn/GSCA/) and Cancer Therapeutics Response Portal (CTRP, https://portals.broadinstitute.org/ctrp/). Pearson analysis showed the correlation between mRNA expression and drug IC_{50}, and FDR value was used to indicate the significance.

2.16. Cell Culture

The BXPC-3 cell line (pancreatic cancer cell), HPDE6-C7 cell line (normal pancreas cell), TE-1 cell line (esophageal cancer cell), and HEEC cell line (normal esophageal epithelial cell) were obtained from the American Type Culture Collection Center (ATCC). HPDE6-C7 cells and HEEC cells supplemented with 10% (FBS) in endotoxin-free in DMEM cultivated; BXPC-3 cells and TE-1 cells were maintained in RPMI-1640 in 10% FBS. All human cell lines are certified by STR within three years.

2.17. Real-Time Quantitative PCR (RT-qPCR)

The total RNA from the cells was extracted by RNA easy reagent and then reverse transcribed by Hiscript Ⅲ 1st Strand cDNA synthesis reagent (vazyme, r323-01). The subsequent RT-qPCR reaction was performed using ChamQ SYBR qPCR Master mix (vazyme, q331-02). See Supplementary Table S1 for specific primer information.

2.18. Statistical Analysis

Students' t-test was used to compare the variables between the two groups. Correlation was determined using Spearman correlation. p values<0.05 were considered statistically significant. All statistical analyses were performed using R software (2022.07.2).

3. Results

3.1. Differential expression and risky score of RNA Modification Writers in pan-cancer

To explore the potential role of RMWs, they were evaluated with cancer sample data from TCGA and normal sample data from GTEx by differential expression analysis across pan-cancer (Fig. 1A). We discovered that most RMW genes had different expressions across different cancer types, and more than half of them are highly expressed in cancer. Among them, CPTF2 and CPSF3 exhibited high expression levels across a wide range of cancers, whereas ADARB1 and ADARB2 demonstrated low expression levels in nearly all cancer types. RT-qPCR experiments verified the expression, and results showed that the high expression of ADAR, TRMT10 and CPSF2 in PAAD and ADAR,

TRMT6, TRMT10, NUDT21, CPSF3, CSTF1 and CSTF2 in ESCA (Fig. 1B).

Further, we explored the relationship between RMWs and prognosis. To identify risky genes, we used univariate Cox regression to analyze the relationship between RMWs and prognosis (Fig. 1C). To visually measure the magnitude of every gene's risk, we developed a risk score to assess. The number of differentially expressed genes obtained by Cox regression analysis using expression and survival data was counted in Pan cancer (Fig. 1D). In order to explore the relationship between RMWs, we analyzed the correlation between the 26 RMWs, and found that most of the RMW expressions were positively correlated (Fig. S1A). At the same time, we also found positive correlations between RMWs not only in the same category, but also between different types of RMWs.

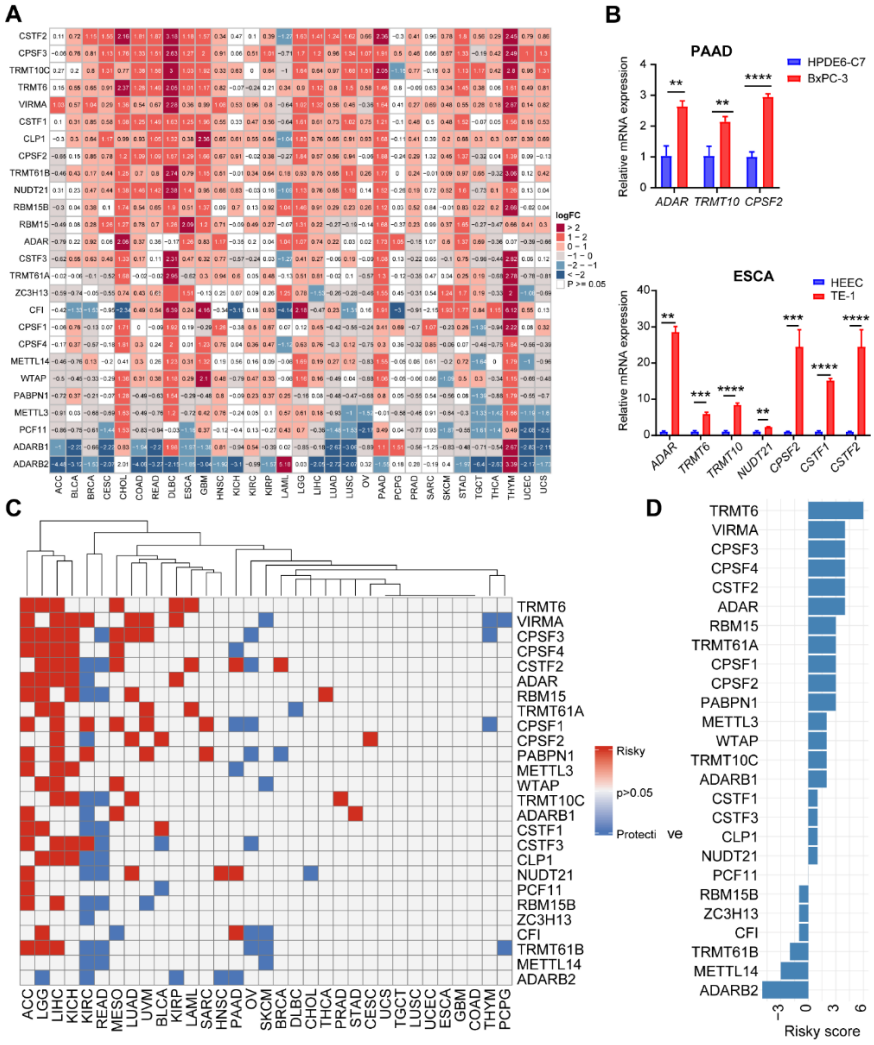


Figure 1. Differential expression and a risk score of RMWs between tumor and normal samples. (A). Differential expression of RMWs between tumor and normal samples in pan-cancer. The color of each box represents the size of the logFC value. White boxes represent p value > 0.05, which has no significant difference. Generally, logFC>1 means fold change of more than 2, which is regarded as the screening condition; logFC> 0: gene expression in cancer is higher than that in normal tissues; logFC < 0: gene expression in normal tissues is higher than that in cancer tissues. The number in the box is the fold change of cancer tissue compared with the normal tissue of the corresponding gene in cancer. (B). RT-qPCR experiment verified the high expression of ADAR, TRMT10, and CPSF2 in PAAD and ADAR, TRMT6, TRMT10, NUDT21, CPSF3, CSTF1and CSTF2 in ESCA. (C). The heatmap showed the univariate Cox regression analysis of RMWs in pan-cancer. Gray indicates p > 0.05; red indicates p < 0.05, HR > 1, implying that the gene was a risky factor in this cancer and blue indicates p < 0.05, HR > 1, implying that the gene was a protective factor in this cancer. (D). Construction of risk score with significant genes in pan-cancer by Cox regression. If a gene was included as a risky factor, then the risk score plus one point. If a gene was included as a protective factor, then the score was minus one point, and the final score was the risk score.

In conclusion, our results showed that most RMWs are highly expressed in cancer and are positively correlated with other RMWs. The high expression of most RMWs is risky in cancer, and the crosstalk between RMWs may have a potential impact on tumorigenesis and development. Our results can be linked with some previous reports, such as m6A writer mettl14 inhibiting colorectal cancer[57]; TRMT6 promoting liver cancer progression through PI3K/AKT signaling pathway[58].

3.2. Genetic alterations of RNA modification writers in pan-cancer

To determine the genetic alterations of RMWs in pan-cancer, we evaluated the variation, mutation and copy number variation of 26 RMWs using the data based on cBioportal website. Variation in the RMW genes in pan-cancer, including mutations, fusions, expansions, deletions, and multiple variants (Fig. 2A). Among cancers, BRCA was alternated the most frequently, second in OV and third in LUAD. As Figure 2A shown, most genes are alternated in cancers. Amplifications occurred the most frequently, with mutations the second of all alteration types. We speculate that different alteration types and frequencies of RMWs may be responsible for the difference.

Since mutations were the second most of alteration, we analyzed mutation types of RMWs deeply in pan-cancer, including variant classification, variant type, SNV class, variants per sample, variant classification summary, and top 10 mutated genes (Fig. 2B-E). In 7 types of variant classification, we found missense mutation was the most frequent mutation. After that, we followed SNP and DEL counts to analyze the variant type of RMWs, including SNP (single nucleotide polymorphism), ONP (oligonucleotide polymorphisms), INS (insertion mutations), DEL (deletion mutations), where the frequency of SNP was the highest. The result showed that C>T, C>A, T>C were the top three mutations with the highest number of occurrences. Oncoplot shows the mutation distribution of RMWs and the classification of alteration types (Fig. 2F). The top 10 mutated genes were ZC3H13, VIRMA, PCF11, CPSF1, ADARB2, ADAR, RBM15, CFI, ADARB1, CSTF3, respectively. Then, we analyzed RMWs related SNP data to detect the frequency and variant types in each cancer subtype. Fig. S1B presents the mutation distribution of the top 10 mutated genes from inputted gene set in sample set and also provides the classification of SNV types.

3.3. Copy number variation analysis of RMW genes

To investigate the reasons for high expression of RMWs, we analyzed the relationship between RMW expression and CNV, methylation, and regulation of miRNAs. Among them, copy number variation (CNV), especially copy number amplification, is one of the main reasons for high expression of cancer genes. To investigate the specific situation of CNVs, we used CNV data from RMWs in the TCGA database. The distribution of CNV pie charts shows that the main types of CNVs in 33 types of cancer are heterozygous amplification and deletion (Fig. 3A). Figure 3B shows the correlation between CNV and mRNA expression. The association between mRNA expression and CNV percentage in samples was based on Spearman's correlation coefficient. The heterozygous CNV map displays the percentage of heterozygous CNVs, including the percentage of amplification and deletion in each cancer (Fig. S2A). Figure provides the profile of heterozygous CNV of inputted genes in the selected cancers. Similar results were gained in the Homozygous CNV profile (Fig. S2B). We found that some RMWs had high CNV frequencies in certain cancers. For example, ZC3H13 had high CNV frequency in BRCA, OV, and LUAD. We also observed that some RMWs had positive or negative correlations between their CNV percentage and mRNA expression. For instance, WTAP had a positive correlation between CNV percentage and mRNA expression in BRCA, while ADARB2 had a negative correlation in OV. These results suggested that the genetic alteration of RMWs may be the reason for the expression of RMWs, which play an important role in cancer progression.

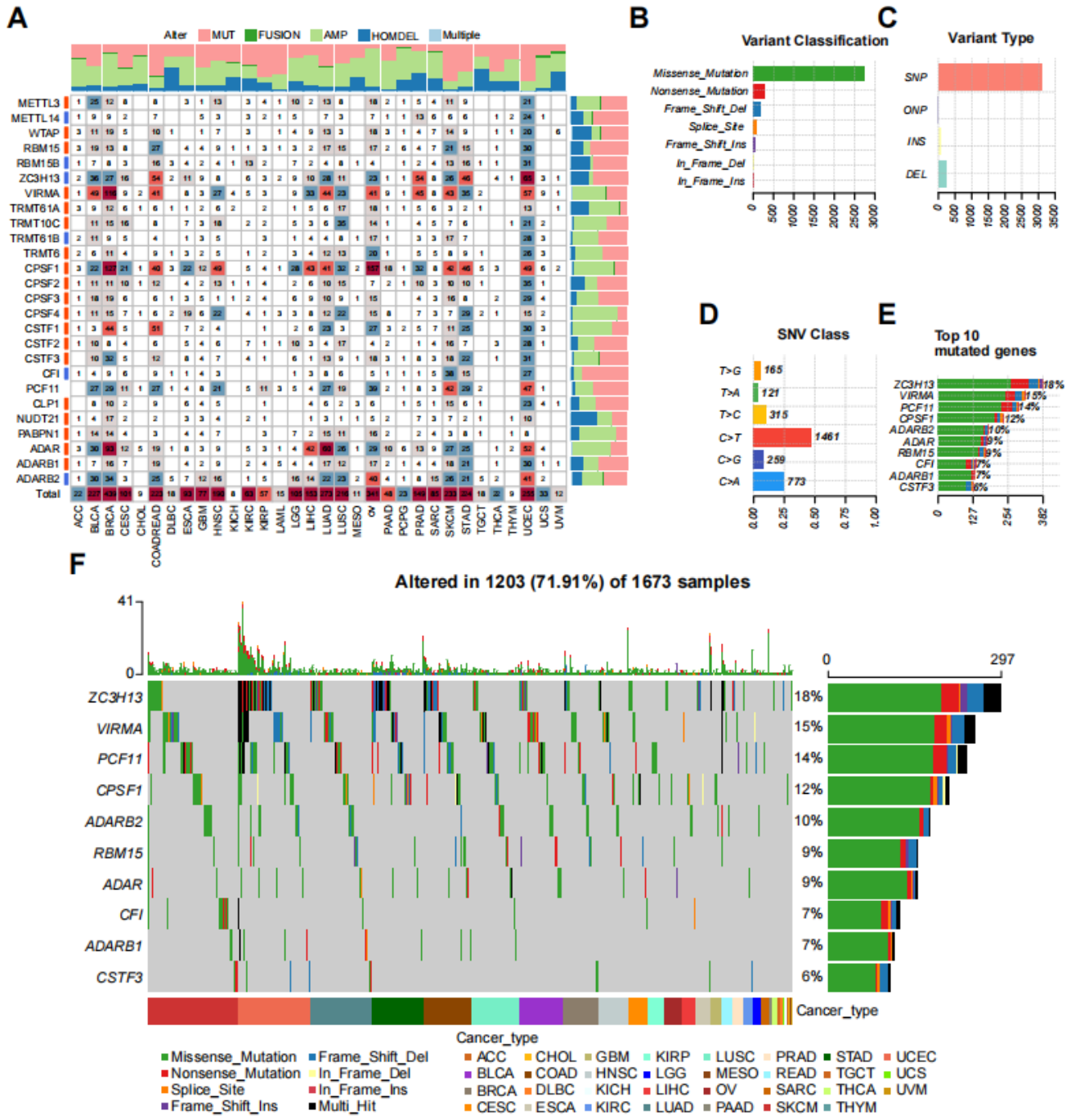


Figure 2. Variation of RMWs. (A). Variation in the RMWs in pan-cancer, including mutations, fusions, expansions, deletions, and multiple variants. The sample size of the genetic variant is indicated by color in boxes. Boxes were displayed in gray when the number of variant samples was greater than 10; greater than 20 was colored orange; greater than 40 was colored dark red. The color at the right of the RMWs means the variant frequency of the gene, red means the variant occurs in more cancers and blue means variant occurs in fewer cancers. (B). Variant classification: the count of each type of deleterious mutation of inputted gene set in selected cancer types. (C). Variant type: the count of SNP and DEL of inputted gene set in selected cancer types. (D). SNV class: the count of each SNV class of inputted gene set in selected cancer types. (E). Top 10 mutated genes: the count and percentage of variants in top 10 mutated genes. (F). Summarizes the frequency of deleterious mutations in selected cancer types. Oncoplot shows the somatic landscape of top 10 alternative RMW in pan cancer. The waterfall plot shows mutation information for each gene for each sample.

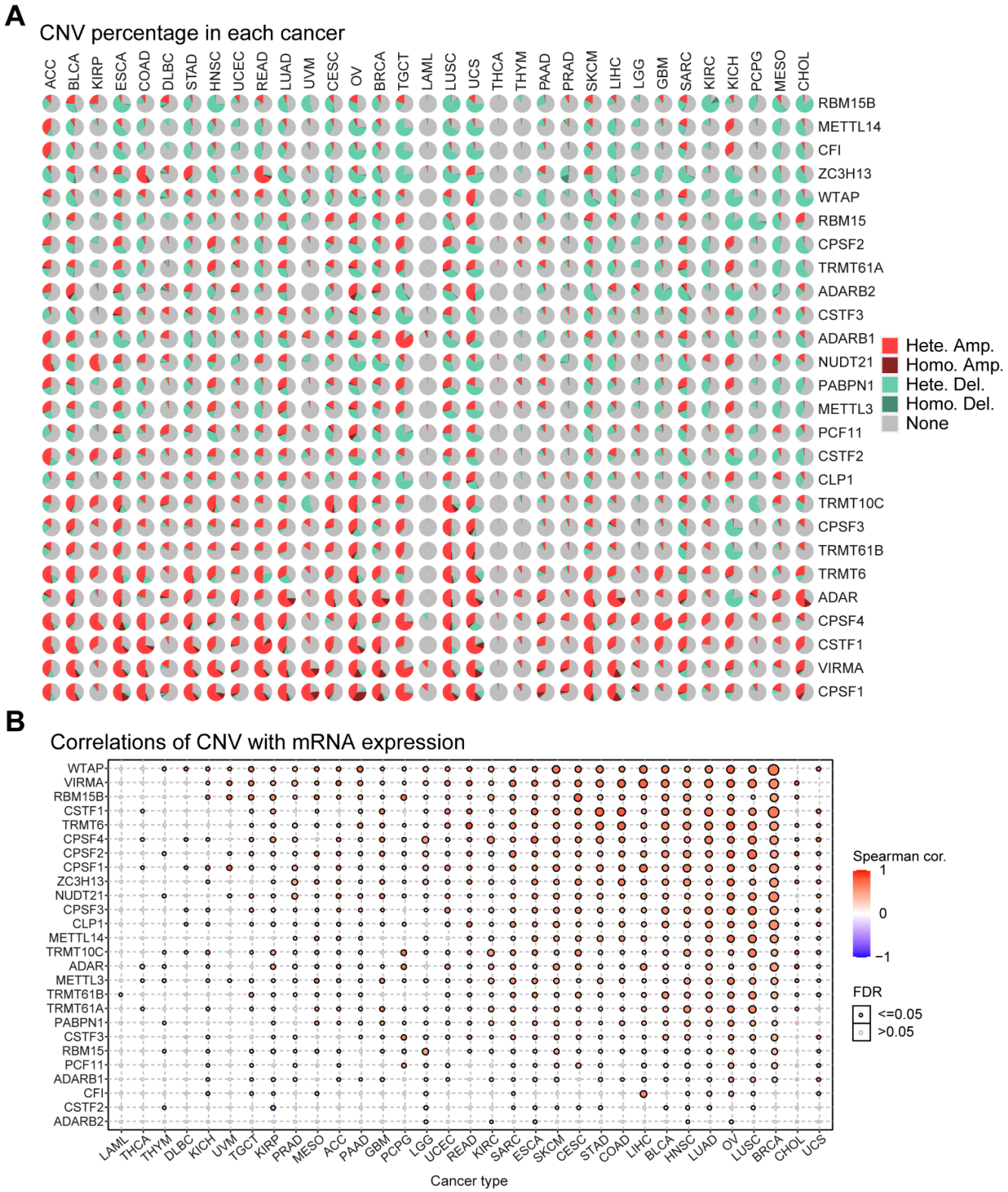


Figure 3. Copy number variation analysis of RMW genes. (A). Pie plot summarizes the CNV of inputted genes in the selected cancer types. Hete Amp, heterozygous amplification; Hete Del, heterozygous deletion; Homo Amp, homozygous amplification; Homo Del, homozygous deletion; None, no CNV. (B). Correlation between CNV and gene expression by non-parametric Spearman correlation test. Red represents positive correlation, blue represents negative correlation, circles represent FDR<=0.05, FDR: false discovery rate.

3.4. Methylation analysis of RNA modification writers

RNA methylation is a key mechanism that modulates gene expression by adding methyl to RNA molecules. In order to explore the epigenetic regulation, we analyzed the methylation of RMWs. As shown in Fig. 4A, the methylation of RMWs in different tumors was highly heterogeneous. These results indicate that most RMWs might be methylation-regulating genes. Fig. 4B shows the correlation between methylation and RMWs expression. The correlation analysis between methylation and mRNA expression showed that the expression levels of most genes were negatively correlated with their methylation levels. Our analysis revealed that the methylation level of most RMWs was negatively correlated with their mRNA expression, suggesting that methylation may act as a repressive mark for gene transcription. Compared with normal samples, the methylation degree of some tumor samples was significantly changed. For example, ZC3H13, VIRMA, PCF11, CPSF1, ADARB2, and ADAR were hypermethylated in tumors, while TRMT10C, TRMT6, TRMT61B, METTL14, and METTL16 were hypomethylated in tumors, which are consistent with previous studies that reported the differential methylation of RMWs in cancer.

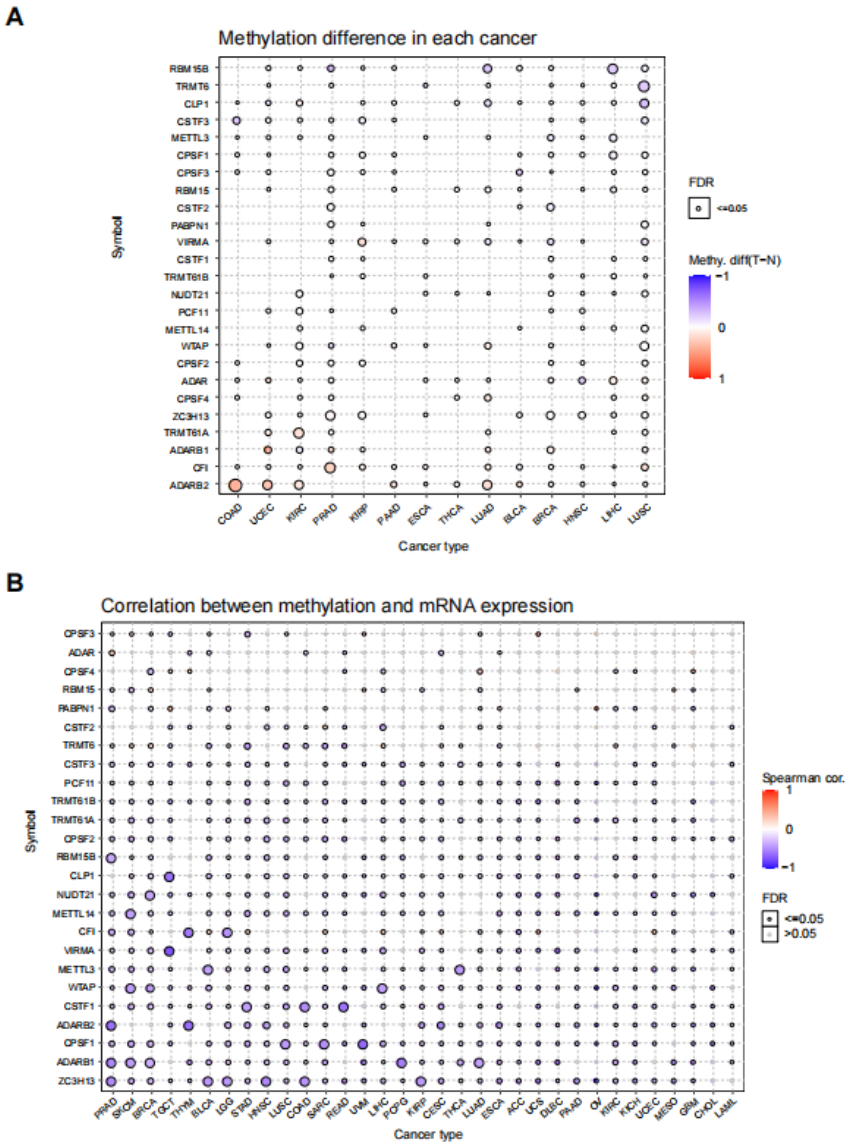


Figure 4. Methylation analysis of RMW genes. (A). Methylation difference between tumor and normal samples of inputted genes in the selected cancers. Circles represent FDR<=0.05, FDR: false discovery rate. (B). Correlation between methylation and mRNA expression in pan-cancer by non-parametric Spearman correlation test.

3.5. Construction of transcription factors and miRNA regulatory network

To better understand the mechanism behind RMWs, a gene regulatory network was constructed. As shown in Fig. 5, results suggested that miRNA regulation of RMWs expression may be related to cancer progression. Some reports have confirmed the relationship in the regulatory network, such as mir-378 downregulated the RNA editing of ADAR, leading to the variability of constitutive liver expression [59]; *METTL3* inhibited thyroid cancer progression through REL-mediated neutrophil infiltration[60]. We also identified that some transcription factors or miRNAs were shared by different RMWs, suggesting a possible cross-talk between different RNA modification pathways. For example, *MYC* was a common transcription factor for 12 RMWs, and hsa-miR-181d was a miRNA that interacted with 3 RMWs simultaneously.

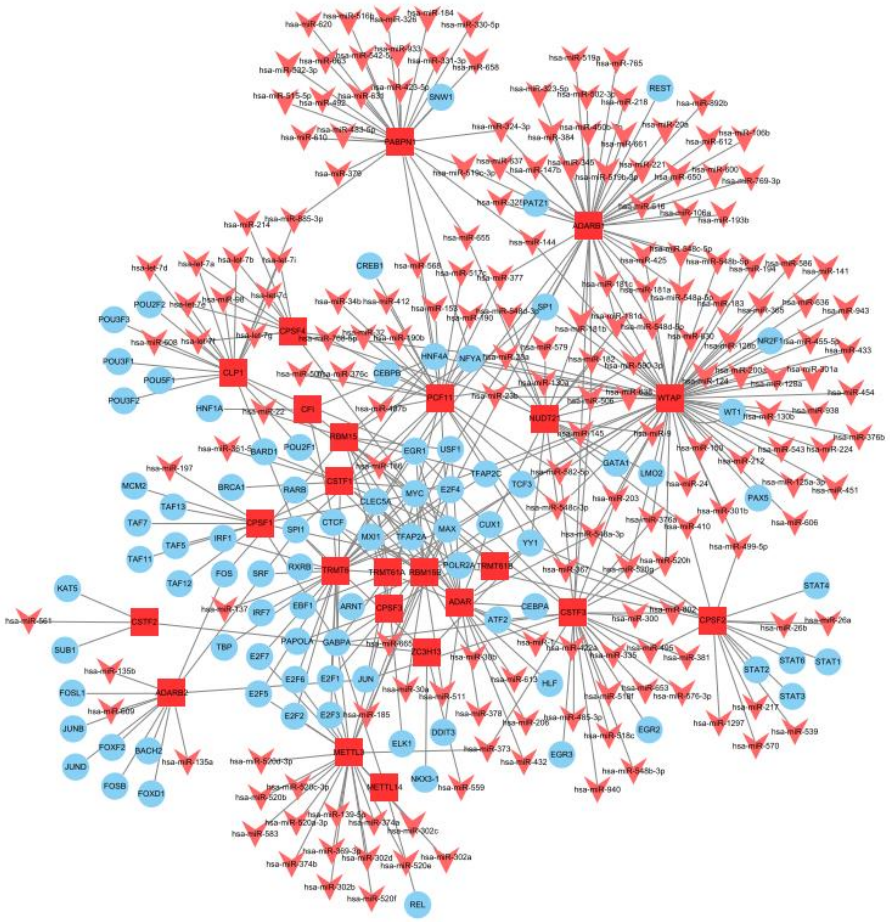


Figure 5. Gene regulatory interaction network construction. Transcription factors and miRNA-regulated RMW genes were analyzed using Regnetwork database and visualized using Cytoscape software (triangle for miRNA, red square for RMW genes, others for transcription factors).

3.6. Construction of RNA Modification Writer score and expression and prognosis analysis

In view of the complexity of RNA modification, we constructed a scoring model to quantitatively evaluate RNA modification. Fig. 6A showed the RMW score in 33 tumors. Fig. S3A plots demonstrated differences in RMW score in paired cancer samples and adjacent normal samples. Among them, the score of samples was higher in cancers such as BLCA, BRCA, COAD, ESCA, HNSC, KICH, LIHC, LUAD, LUSC, PRAD, READ, STAD, and UCEC than in normal samples, while normal samples were higher in KIRC.

To validate the association between RMW score and patient outcome in different tumors, univariate COX

regression was used for analysis (Fig. 6B). Four survival models OS (overall survival), DSS (disease-specific survival), DFI (disease-free interval), and PFI (progression-free interval) were constructed by R package survminer and survival. High RMW scores were significantly associated with poor prognosis of patients in CESC, LIHC and PRAD by OS; in KICH, PRAD, CESC by DSS; in KIRP, PRAD, LIHC, CESC, PCPG, ACC by DFI; in KIRP, CESC, PRAD, ACC, LIHC by PFI. High scores of RMW score can be regarded as risk factors in above cancers. Summarizing the above results, we found when it mentions to RMW score in cancers such as PRAD, LIHC, CESC, and KIRP, it was considered as a risky factor significantly by multiple methods, while a protective factor in OV. Interestingly, we found that the RMW score in PRAD was not only significantly associated with high mRNA expression in tumors, but also correlated with poor prognosis. This suggested that RNA modification may plays a more important role than others in PRAD.

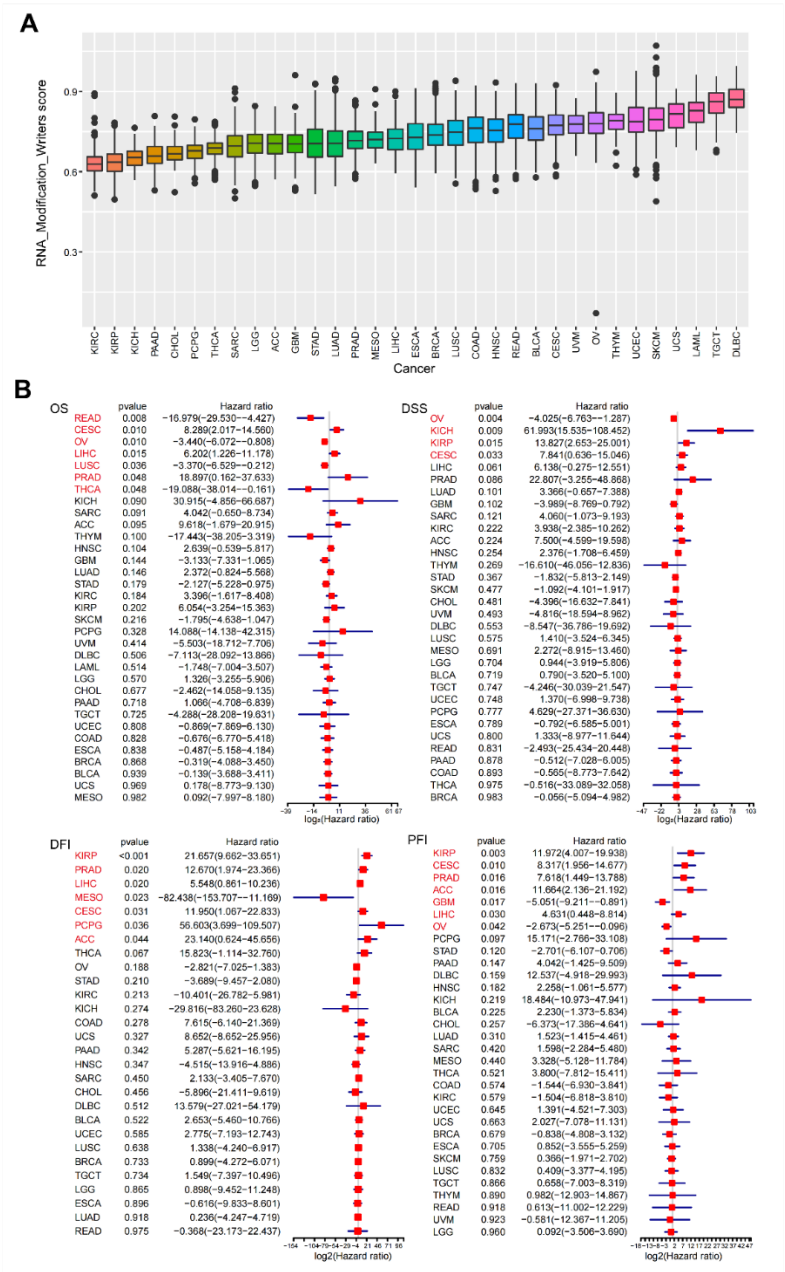


Figure 6. Construction of RNA Modification Writers score and survival analysis of the RMW score in pan-cancer. (A). RMW score in 33 tumors, with the scores increasing sequentially from left to right. (B). Survival analysis of the RMW score in pan-cancer. OS, DSS, DFI, PFI survival analysis models by univariate Cox regression in pan-cancer. Red shows the tumors with a signature p-value, and the abscissa represents log2 (Hazard ratio).

3.7. The GSVA analysis of the RMW score

To further investigate the underlying mechanisms of these RMW in cancer, we performed GSVA to analyze pathway level enrichment of 26 RMW gene sets (Fig. 7A). The top5 pathways with the strongest positive correlation with RMW score in pan-cancer were MYC TARGETS V1, E2F TARGETS, G2M CHECKPOINT, MYC TARGETS V2, and DNA REPAIR. The top5 pathways with the strongest negative correlation with RMW score in pan-cancer were MYOGENESIS, XENOBITIC METABOLISM, COAGULATION, ESTROGEN RESPONSE EARLY, and P53 PATHWAY. We speculated that the RMW score is related to the growth and proliferation of cancer cells mediated by transcription factors, cell cycle and repair of DNA damage which leads to the progression of cancer with these results.

3.8. Tumor microenvironment analysis of RMW score

To gain further insight of RMW score on immune response, we assessed association between RMW score and the immune microenvironment in cancer samples. As shown in Fig. 7B, heatmap of correlation between RMW scores with tumor purity, immune score, ESTIMATE score, and stromal score. A clear correlation was found between RMW score and tumor microenvironment (immune score and stromal score). There was a significant negative correlation between RMW score and immune score, stromal score, ESTIMATE score, which was consistent with the tumor purity trend of RMW in vast majority of cancers.

Then, we used the correlation analysis between RMW scores and pathways reported in the previous literature[54] to evaluate the tumor microenvironment, including immune-related pathways, matrix/metastasis-related pathways and DNA damage repair related pathways (Fig. 7C). We found that RMW scores were positively correlated with DNA damage repair pathways in the majority of cancers. However, an overactivated DNA damage repair system would promote tumor cell invasion and metastasis. Altogether, these data indicate that majority of above pathways may play key roles in connecting RMWs with tumor immunity in these cancers.

3.9. Immune cell infiltration and correlation analysis of related immune genes

Immune cells played a crucial role in the tumor microenvironment, and their infiltration is closely related to the occurrence and development of tumors. To explore the correlation between RMWs and tumor immune cell infiltration, we used online database ImmuCellAI and TIMER2 to analyze the correlation. As shown in Fig. 7D, most of the correlation between the expression of RMW genes and the infiltration of immune cells was negative. The top three immune cells negatively correlated with RMW gene were NK cells, MAIT cells, and Tfh cells. RMW score negatively correlated with multiple immune cell infiltrates were found in Fig. S3B by six different immune algorithms (EPIC, TIMER, CIBERSORT, QUANTISEQ, MCPCOUNTER, XCELL) in the TIMER2 database. In both databases, we found RMW score was negatively correlated with multiple immune cell infiltrates. To sum up, we concluded that tumors with high RMW score may be immune-desert tumors without enough response to immunotherapy.

We investigated the correlation between immune cells and related immune genes in human cancers, including immunoactivated genes (Fig. 8A), MHC genes (Fig. 8B), chemokines (Fig. 8C), and chemokine receptor genes (Fig. 8D). As shown in Figure 13, the RMW score was mostly negatively correlated with these immune-related genes, however, the opposite result appeared in THCA and CHOL. The correlation of immune-related genes was consistent with the results of immune cell infiltration, so we speculated that RMW inhibited immune-related gene expression when it was highly expressed. Therefore, the RMW score has the potential to be used as an important factor in determining whether a patient is suitable for immunotherapy.

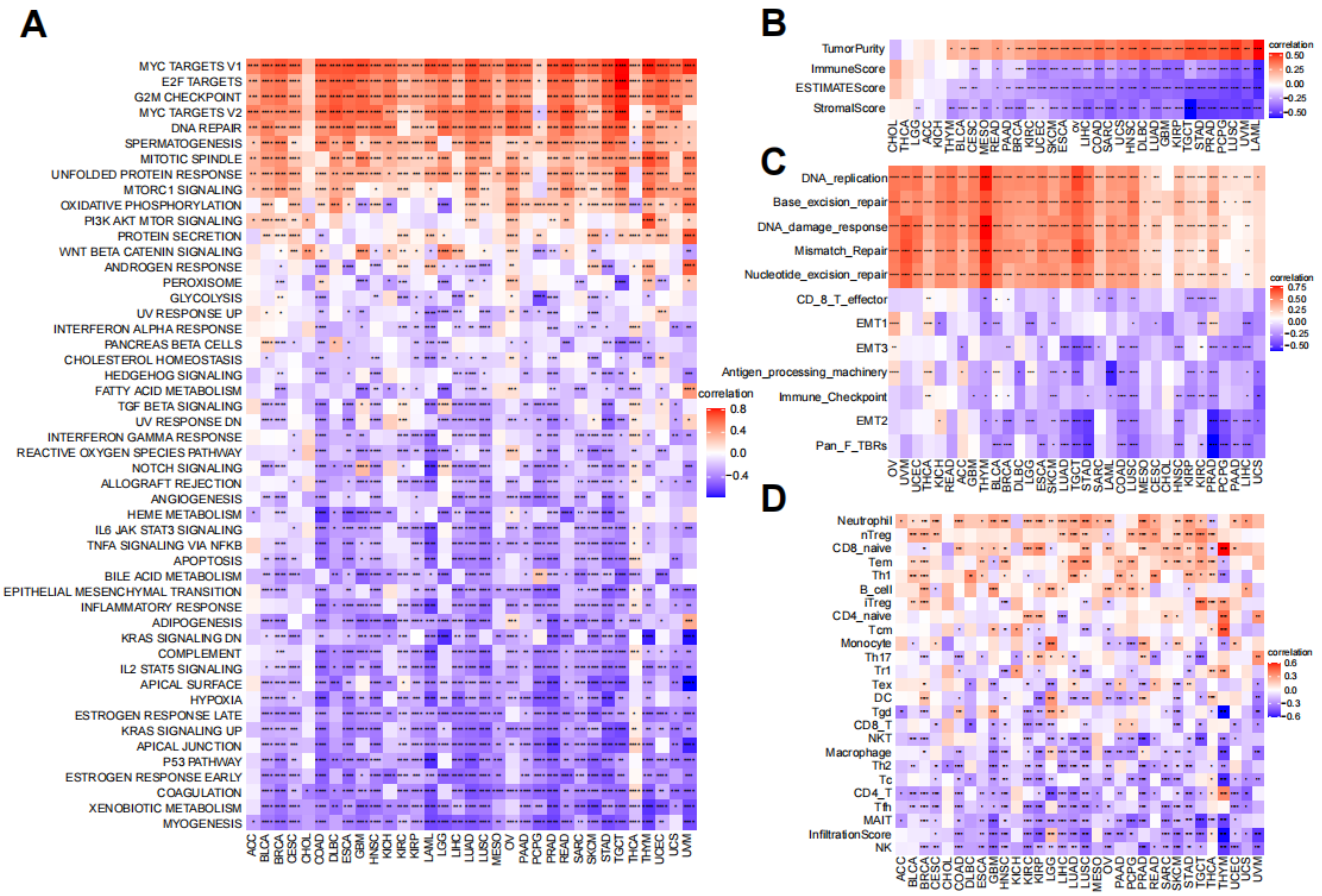


Figure 7. The GSVA analysis of the RMW score and correlation between immune cell infiltration and RMW score. (A). Correlation between the RMW score and the cancer hallmark pathway. Red represents positive correlation, blue represents negative correlation, * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. (B). Heatmap of correlation among RMW score and tumor purity, immune score, ESTIMATE score, stromal score. (C). Heatmap of correlation between RMW score and immune-related pathways, stroma and metastasis pathways, DNA damage repair related pathways. (D). Heatmap of immune cell infiltration in pan-cancer from the ImmuCellAI database.

3.10. Drug Sensitivity Analysis of RNA Modification Writers

To investigate the effects of different treatments on patients in high and low RMW score groups, we analyzed the prognosis of patients from different treatment data. The Fig. 9A-D data was referred from the previous research[61], containing patients treated with immunotherapy (Fig. 9A, B) and targeted agents (Fig. 9C, D). As shown in Fig. 9A, the overall survival analysis of KIRC patients with targeted therapy mTOR inhibitor demonstrated that the low-score group had significantly worse survival than the high-score group. The disease progression histogram after everolimus treatment showed the proportion of patients with remission and progression (Fig. 9B, CR: complete response; PR: partial response; PD: progressive disease; SD: stable disease). As shown in Fig. 9C, the overall survival analysis of KIRC patients treated with nivolumab immunotherapy demonstrated that the low-score group had significantly worse survival than the high-score group. With this treatment, 20% of patients in the high-score group responded and 23% in the low-score group responded (Fig. 9D). As mentioned in the above four figures, it indicated that the effect of immunotherapy is better than targeted therapy in high-score group. As shown in Fig. 9E, the progression-free survival analysis of advanced non-small cell lung carcinoma patients treated with anti-PD-1/PD-L1 immunotherapy demonstrated that the low-score group had significantly worse survival than the high-

score group. The disease progression of all patients in the high-score group could not control by the condition of immunotherapy, while 33% of the patients in the low-score group were controlled with anti-PD-1/PD-L1 immunotherapy (Fig. 9E, F). As shown in Fig 9G, the overall survival analysis of metastatic urothelial cancer patients treated with immune checkpoint blockade demonstrated that the low-score group had significantly worse survival than the high-score group. There were 11% of patients in the high-score group responded and 19% in the low-score group responded (Fig. 9G, H). Generally speaking, the low-score group showed better effects and survival in immunotherapy than targeted therapy. The analysis of clinical samples in this section further revealed that the RMW score may act as a potential biomarker for immunotherapy. After finding low-score group has a poor effect on targeted treatment, we investigated the role of RMWs on targeted therapy (Fig. 9I, J). Pearson correlation analysis was performed to get the correlation between mRNA expression and drug IC_{50}. Most RMW expression was negatively correlated with drug IC_{50}. However, IC_{50} of 17-AAG, trametinib was positively correlated with the majority of RMWs expression. Interestingly, the expression of CFI is positively correlated with IC_{50}, which is contrary to other RMWs, meaning that the high expression of CFI may be associated with drug resistance. These results suggest that the low expression of most RMWs may mediate the drug resistance, which is consistent with the poor efficacy of the low-score group treated with targeted therapy.

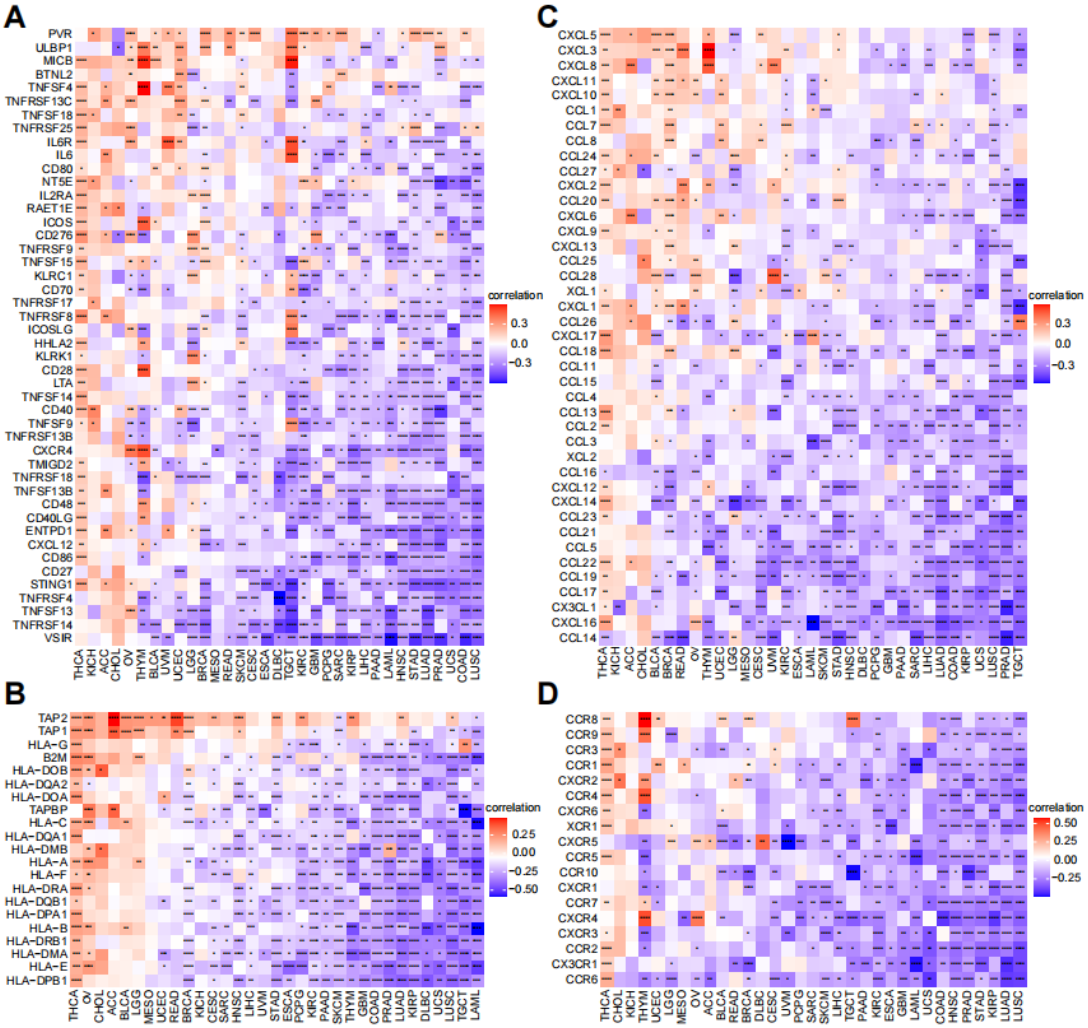


Figure 8. Analysis of immune related genes correlation in pan-cancer. (A). Correlation between immunoactivated genes and RMW score. (B). Correlation between MHC genes and RMW score. (C). Correlation between chemotactic factor genes and RMW score. (D). Correlation between chemokine receptor genes and RMW score.

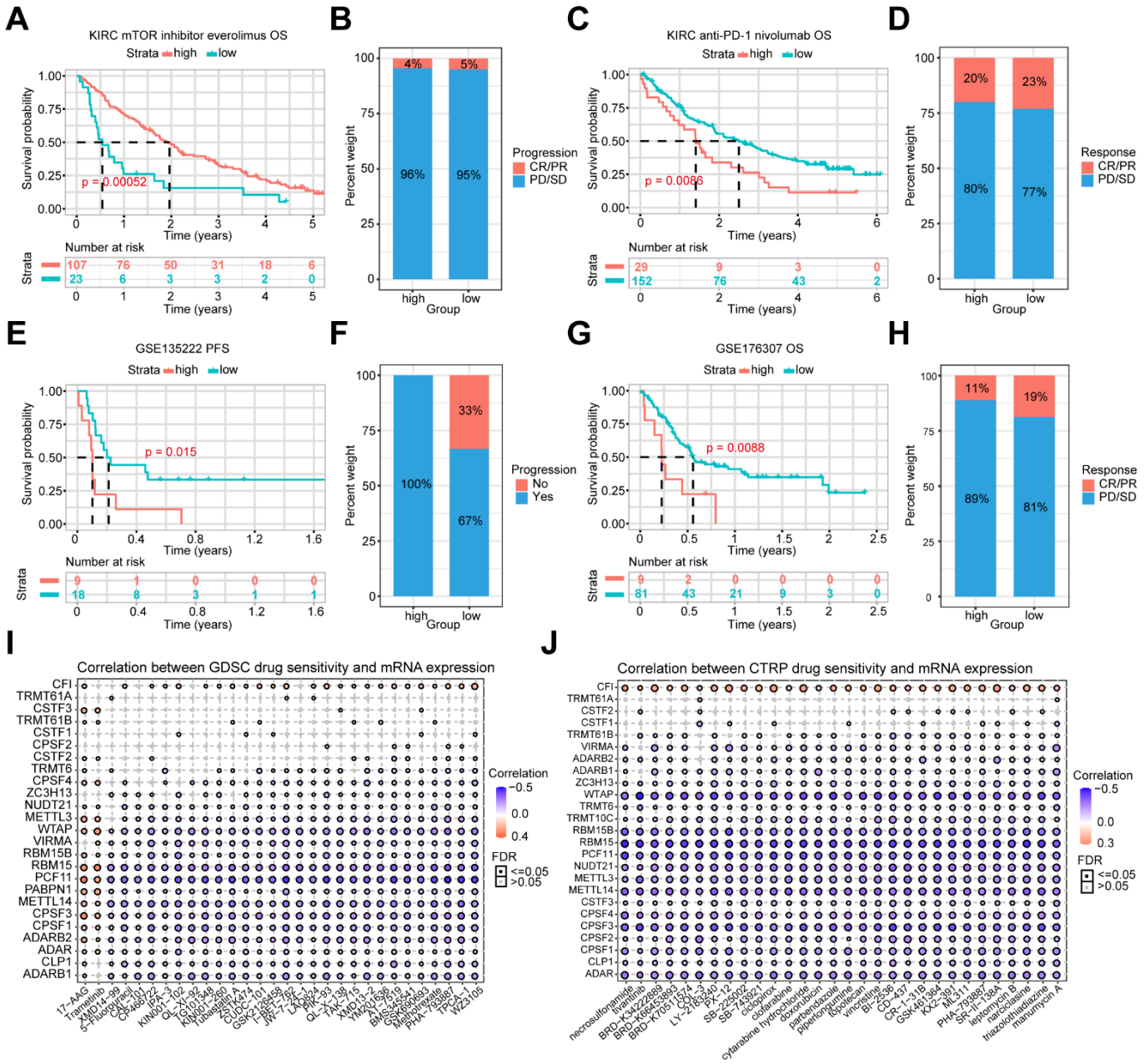


Figure 9. The effect of the RNA Modification Writer score on the prognosis of patients with immunotherapy and targeted therapy and prediction of targeted therapeutic drugs. (A). Overall survival analysis of KIRC patients treated with targeted therapy. (B). Histogram of disease progression after everolimus treatment. (C). Overall survival analysis of KIRC patients treated with immunotherapy. (D). Histogram of disease progression after nivolumab treatment. (E). Progression-free survival analysis of NSCLC patients treated with anti-PD-1/PD-L1 from GSE135222. (F). Histogram of disease progression after immunotherapy with anti-PD-1/PD-L1. (G). Overall survival analysis of patients with EGFR3-altered metastatic urothelial cancer treated with ICB from GSE176307. (H). Histogram of disease progression after immunotherapy with ICB. (I). Figure summarizes the correlation between gene expression and the sensitivity of GDSC drugs (top 30) in pan-cancer. (J). Figure summarizes the correlation between gene expression and the sensitivity of CTRP drugs (top 30) in pan-cancer.

4. Discussion

RNA modification is an important epigenetic mechanism that controls gene expression, and it is involved in various biological processes and human diseases, especially cancer. Adenosine alterations are catalyzed by RNA modification writers, such as m6A, m1A, APA, and A-to-I editing. In this study, we comprehensively analyzed the molecular and clinical characteristics of 26 RMWs in 32 cancers.

We found that compared with normal samples, most RMW genes were highly expressed in cancer samples. After RT qPCR experiments, we verified that ADAR, TRMT10, CPSF2 and other genes were highly expressed in pancreatic cancer and colorectal cancer cells. Integrating the sample expression and survival data, we also identified some RMWs as risk factors, such as TRMT6, VIRMA, CPSF3, CPSF4, CSTF2, ADAR, and protective factors, such as ADARB2, METTL14, TRMT61B. The high expression of these RMW genes has been shown to promote cancer in previous reports. For example, TRMT6 promotes hepatocellular carcinoma progression through the PI3K/AKT signaling pathway; CPSF4 promotes triple negative breast cancer metastasis by upregulating MDM4; ADAR edits an editing site of azin1, resulting in the s367g substitution at this site, which changes the structure and localization of the protein and endows the protein with a greater cancer promoting function in HCC. Our study also provides some novel insights into the role of RNA modification in cancer. For instance, we revealed that some RMWs had positive correlations with different types of modification writers, such as METTL3 with TRMT6 and ADAR with CPSF4, indicating potential coordination or cooperation between different RNA modification pathways.

Genetic alteration is a term that refers to any change in the genome, which can affect the structure, function, and expression of genes. Cancer cells often have multiple genetic alterations that confer them with advantages over normal cells, such as increased proliferation, survival, invasion, and resistance to therapy. Genetic alterations are common events in cancer that affect the expression and function of genes involved in various cellular processes. We analyzed the genetic alterations of RMWs in pan-cancer and found that amplification and mutation were the most common types of variants, while BRCA, OV, and LUAD had the highest frequency of alterations in cancer, and ZC3H13, VIRMA, and PCF11 had the highest frequency of alterations in RMWs. We found that mutations were the second most frequent type of alteration after amplifications and that missense mutations were the most common subtype. We also found that SNPs were the most prevalent variant type, and that C>T, C>A and T>C were the top three SNV classes.

To further investigate the reasons for the high expression of RMWs, we analyzed the copy number variation, methylation of genes and the regulation of transcription factors and mRNAs on RMWs. We analyzed the CNV data of RMWs in pan-cancer, and found that heterozygous amplifications and deletions were the main CNV types. We also found that some RMWs had high CNV frequencies in certain cancers. For example, ZC3H13 had high CNV frequency in BRCA, OV and LUAD. Our analysis revealed that the methylation level of most RMWs was negatively correlated with their mRNA expression, suggesting that methylation may act as a repressive mark for gene transcription. In addition, we observed that the methylation pattern of RMWs was highly heterogeneous in different tumors. Compared with normal samples, the methylation degree of some tumor samples was significantly changed. For example, ZC3H13, VIRMA, PCF11, CPSF1, ADARB2 and ADAR were hypermethylated in tumors, while TRMT10C, TRMT6, TRMT61B, METTL14 and METTL16 were hypomethylated in tumors, which are consistent with previous studies that reported the differential methylation of RMWs in cancer. In addition, we investigated the regulation of RMWs by transcription factors and miRNAs. We identified that some RMWs were regulated by multiple transcription factors or miRNAs, indicating a complex regulatory network for RNA modification. We also identified that some transcription factors or miRNAs were shared by different RMWs, suggesting a possible cross-talk between different RNA modification pathways.

To further analyze the heterogeneity and complexity of RMW, we constructed a scoring model to quantify the mode of action of RMW. First, we performed the RMW score for each tumor, and compared the RMW score between

each cancer sample and its corresponding normal sample. Among them, the RMW score of most cancer samples, such as BLCA, BRCA, COAD, ESCA, HNSC, KICH, LIHC, LUAD, LUSC, PRAD, READ, STAD, UCEC, is higher than that of normal samples. The RMW score and the prognosis of each cancer were analyzed in pan-cancer, and Cox regression analysis was carried out using four different models: OS, DSS, DFI, PFI. Next, we analyzed the correlation between RMW score and tumor related pathways, immune related pathways and immune related cells (Immune activating genes, MHC genes, chemokine genes, chemokine receptor genes) to examine the pathways and gene enrichment levels of 26 RWMs. We found that the five pathways with the strongest positive correlation with RNA modification writer score in pan-cancer were MYC target V1, E2F target, G2M checkpoint, MYC target V2, and DNA repair. These results suggest that RNA modification writer score may be related to transcription factor mediated growth and proliferation of cancer cells, cell cycle and repair of DNA damage, thus leading to the occurrence and development of cancer.

To gain further insight of the RMW score on immune response, we assessed association between RNA Modification Writer score and the immune microenvironment in cancer samples. We found a clear correlation between RNA Modification Writer score and tumor microenvironment (immune score and stromal score). There was a significant negative correlation between RNA Modification Writer score and immune score, stromal score and ESTIMATE score, and a significant positive correlation between RNA Modification Writer score, which was consistent with the tumor purity trend of RMW in vast majority of cancers. We also assessed the tumor microenvironment, including immune-related pathways, matrix/metastasis-related pathways and DNA damage repair related pathways. We found that RMW scores were positively correlated with DNA damage repair pathways in the majority of cancers. However, overactivated DNA damage repair system would promote tumor cell invasion and metastasis. Altogether, these data indicate that some of these pathways may play key roles in connecting tumor RNA Modification Writer with tumor immunity in these cancers.

To investigate the effects of different treatment on patients in high and low RMW score groups, we analyzed the prognosis of patients from different treatment data. We found that the low RMW score group showed better effects and survival in immunotherapy than targeted therapy. We also found that the RMW score was associated with the therapeutic efficacy of PD-L1 blockade, suggesting the development of potential drugs targeting these RMWs to aid the clinical benefits of immunotherapy.

In order to investigate the role of RMWs on targeted therapy, we integrated drug sensitivity and gene expression profiling data of cancer cell lines from the GDSC and CTRP databases. We performed Pearson correlation analysis to get the correlation between gene mRNA expression and drug IC_{50} and found that most RMWs' expression was negatively correlated with drug IC_{50} by GDSC and CTRP databases. These results suggest that the low expression of most RMW genes may mediate the resistance to chemotherapy and targeted drug therapy, which is consistent with the poor efficacy of low group treated targeted therapy we previously found.

In conclusion, we provided new insights into the role of RMWs in cancer development and progression, and suggested that RMWs can be serve as potential biomarkers for personalized treatment of cancer patients for targeted therapy and immunotherapy.

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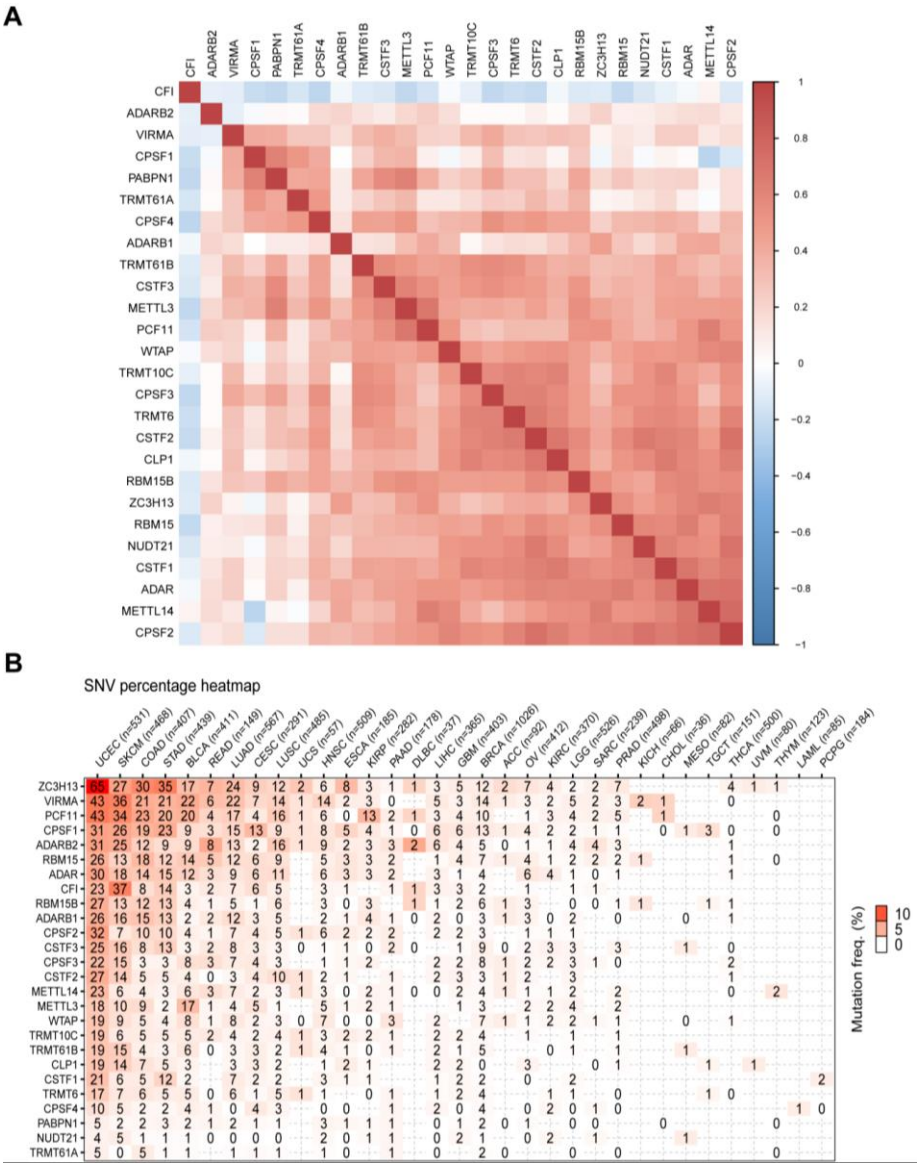
Conflict of interest

The authors declare no competing interests.

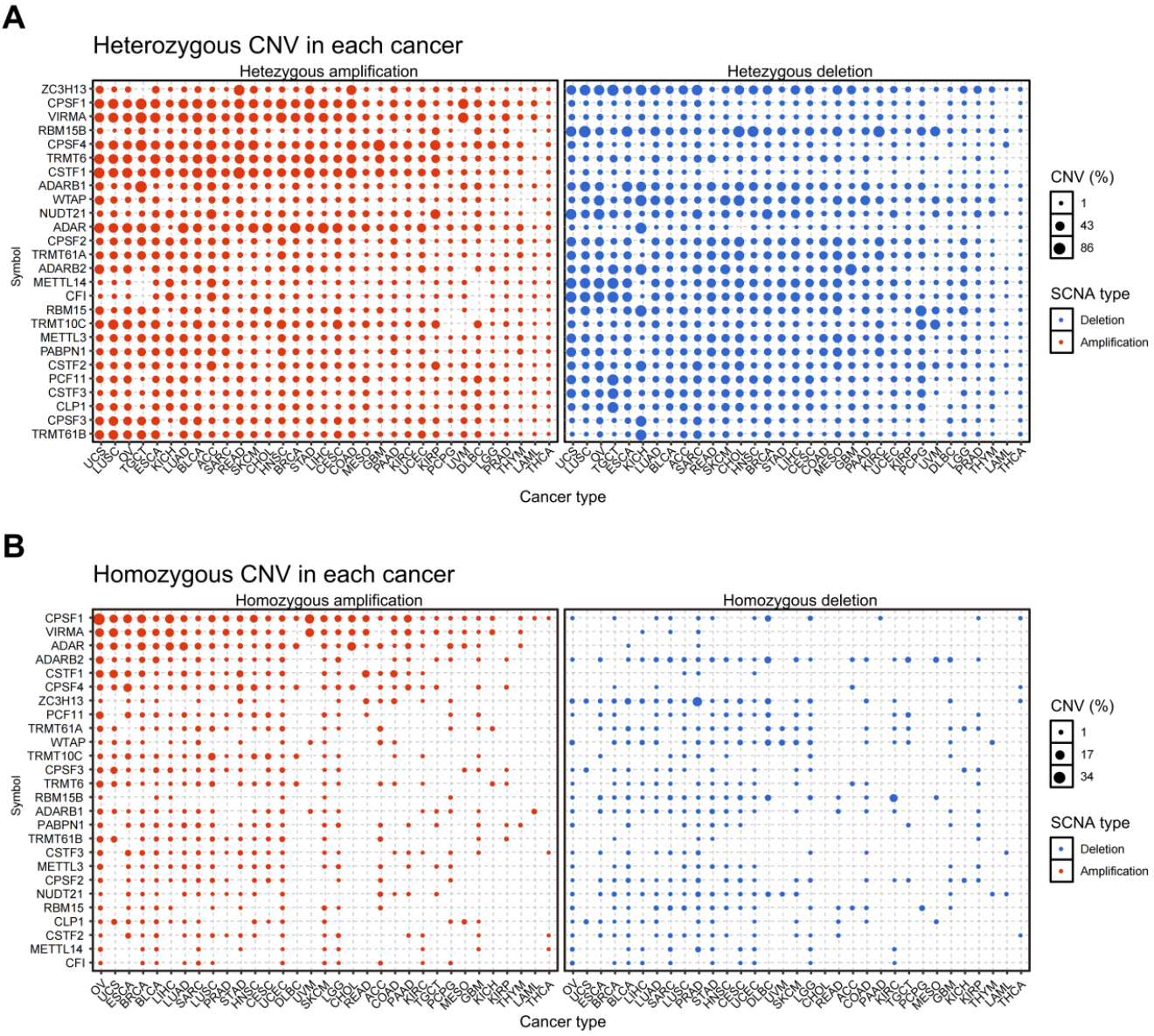
Author contributions

Conceptualization: Peng Yang, Xiao Wang, Didi Wan, Jiayu Ding; Supervision: Peng Yang, Xiao Wang, Didi Wan; Writing-original draft preparation: Jiayu Ding, Hao Shen, Jiaying Ji; Writing - review and editing: Jiaxing Li, Wenbin Kuang, Zhongrui Shi, Dawei Wang, Yuanyuan Chen, Didi Wan, Xiao Wang, Peng Yang.

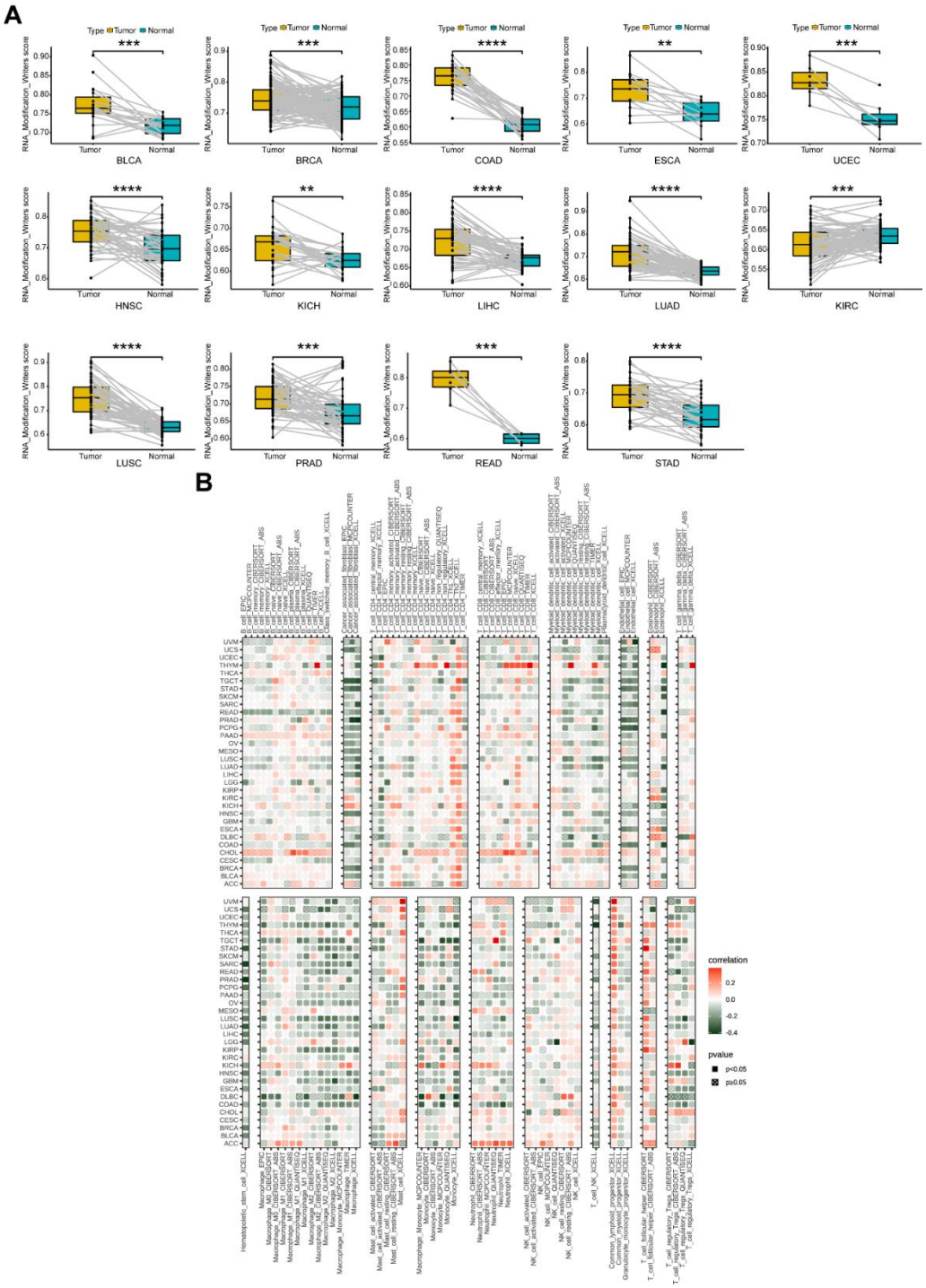
Supplementary



Supplementary Figure 1. Expression correlation and variation of RMWs in pan-cancer. (A). Expression correlation of each two RMWs in pan-cancer. The color of the square represents the correlation coefficient, red represents the positive correlation, and blue represents the negative correlation. (B). Heatmap of the frequency of single nucleotide variation of RMWs in pan-cancer. The color of the square represents the frequency, and the darker the color, the higher the mutation frequency.



Supplementary Figure 2. Homozygous and heterozygous copy number variation of RMWs in pan-cancer. (A). Figure provides the profile of heterozygous CNV of RMW in pan-cancer. (B). Figure provides the profile of homozygous CNV of RMW in pan-cancer. The dot size represents the ratio of copy number variation, blue represents deletion, and red represents amplification.



Supplementary Figure 3. The difference in RMW scores between cancer samples and control normal samples in cancers and correlation with immune infiltration. (A). Differences in RMW score between paired cancer samples and adjacent samples. (B). The correlation between RMW score and immune cell (B cell, class switched memory B, cancer associated fibroblast, T cell CD4 central memory, T cell CD4 effector memory, T cell CD4, T cell CD4 memory actived, T cell CD4 memory resting, T cell CD4 memory, T cell CD4 naive, T cell CD4 non regulatory, T cell CD4 Th1, T cell CD4 Th2, T cell CD8 central memory, T cell CD8, T cell CD8 effector memory, T cell CD8 naive, Myeloid dendritic cell actived, Myeloid dendritic cell, Myeloid dendritic cell resting, Plasmacytoid dendritic cell, Endothelial cell, Eosinophil, T cell gamma delta, Hematopoietic stem cell, Macrophage, Macrophage M0, Macrophage M1, Macrophage M2, Macrophage Monocyte, Mast cell activated, Mast cell resting, Mast cell, Monocyte, Neutrophil, NK cell activated, NK cell, NK cell resting, T cell NK, Common lymphoid progenitor, Common myeloid progenitor, Granulocyte monocyte progenitor, T cell follicular helper, T cell regulatory) infiltration by six models (EPIC, MCPCOUNTER, CIBERSORT, CIBERSORT ABS, XCELL, QUANTISEQ, TIMER), the red square represents the positive correlation, the green square represents the negative correlation, and the solid square represents the correlation has significant difference.

Supplementary Table 1. Primer sequences for genes in RT-qPCR.

Name	Sense (5'-3')	Antisense (5'-3')
GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG
ADAR	ACGGGCCCTCTAGACTCGAGCGCCACCATGAATCC GCGGCAGGGGTATTCCCTC	AGTCACTTAAGCTTGGTACCGATACTGGGCA GAGATAAAAGTTCTTTTC
TRMT6	AGTCACTTAAGCTTGGTACCGAT	CCACCTCCACTCATCAGCAG
TRMT10C	TCAAGCTGCTAGAAACCACTG	TCTGTGCAAAGCACCATCTATT
NUDT21	ACAAGTACATCCAGCAGACGAAGC	AGCCGGTGCTCATGTACAATCAG
CPSF2	ATGACGTCTATTATCAAATTAACTA	TTATACAATGGCATATTGTTCATAT
CSTF1	TCGCCAATGGCCTCATCAAT	TGCATACTGAACTGCGGTGT
CSTF2	ATGACGTCTATTATCAAATTAACTA	TTATACAATGGCATATTGTTCATAT

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